Regulatory changes in the alpha-cardiac actin variants H88Y and F90Δ implicated in early-onset hypertrophic cardiomyopathy

by

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ABSTRACT

REGULATORY CHANGES IN THE ALPHA-CARDIAC ACTIN VARIANTS H88Y AND F90Δ IMPLICATED IN EARLY-ONSET HYPERTROPHIC CARDIOMYOPATHY

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University of Guelph, 2018

Cardiovascular disease (CVD) impacts millions of lives affecting 1 in 12 Canadians over the age of 20 between 2012 and 2013. A commonly inherited CVD called Hypertrophic cardiomyopathy (HCM) is defined by an increase in ventricular wall thickness. Little is known about the molecular pathogenesis of HCM apart from its link to mutations in genes encoding sarcomere proteins, including α-cardiac actin (ACTC). My research focuses on the F90Δ and H88Y ACTC variants in early-onset HCM. Studies have shown that myosin activity is largely unchanged with these ACTC variants. I hypothesized that these ACTC variants adversely affect tropomyosin (Tm) regulation, decreasing contractility. Regulated thin filaments (RTFs), and both myosin ATPase assay and an in vitro motility assays were used to generate pCa50 curves. The RTF calcium sensitivity is affected by F90Δ and H88Y variants. These data will contribute to understanding the molecular pathogenesis of early-onset HCM leading to more specialized treatments.
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<th>Full Name</th>
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<tbody>
<tr>
<td>F90Δ-ACTC</td>
<td>Human α-cardiac actin containing an F90Δ substitution</td>
</tr>
<tr>
<td>H88Y-ACTC</td>
<td>Human α-cardiac actin containing an H88Y substitution</td>
</tr>
<tr>
<td>ABB</td>
<td>Assay base buffer</td>
</tr>
<tr>
<td>ACTC</td>
<td>α-cardiac actin protein</td>
</tr>
<tr>
<td>ACTCl</td>
<td>α-cardiac actin gene</td>
</tr>
<tr>
<td>HMM</td>
<td>Heavy meromyosin</td>
</tr>
<tr>
<td>IVM</td>
<td>In vitro motility</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricle/ventricular</td>
</tr>
<tr>
<td>MHC</td>
<td>Myosin heavy chain</td>
</tr>
<tr>
<td>Ni-NTA</td>
<td>Nickel nitritetriacetic acid affinity chromatography resin</td>
</tr>
<tr>
<td>pCa50</td>
<td>-log of the Ca²⁺ concentration that elicits half maximal activity</td>
</tr>
<tr>
<td>RTF</td>
<td>Regulated thin filament composed of actin, troponin and tropomyosin</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DCM</td>
<td>Dilated cardiomyopathy</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethanol Sepharose® ion exchange chromatography resin</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>HCM</td>
<td>Hypertrophic cardiomyopathy</td>
</tr>
<tr>
<td>His-G4-6</td>
<td>Histidine-6-tagged gelsolin subdomain 4-6</td>
</tr>
<tr>
<td>SCD</td>
<td>Sudden cardiac death</td>
</tr>
<tr>
<td>Sf21</td>
<td><em>Spodoptera frugiperda</em> ovarian clonal isolate 21</td>
</tr>
<tr>
<td>TnT/I/C</td>
<td>Cardiac troponin subunit T/I/C</td>
</tr>
<tr>
<td>Tn</td>
<td>Troponin complex</td>
</tr>
<tr>
<td>Tm</td>
<td>Tropomyosin</td>
</tr>
<tr>
<td>WTrec</td>
<td>Wild-type recombinant human actin</td>
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1. Introduction:

Research Significance

Hypertrophic cardiomyopathy (HCM) is a highly prevalent cardiovascular disease that is defined by unexplained left ventricular hypertrophy. Clinicians classified this disease over 50 years ago, but the underlying cause has yet to be fully explained. The progressive hypertrophy was initially thought to be idiopathic or a secondary characteristic of metabolic disorder. Molecular genetic studies have led to the discovery of mutations linked to this disease. There is still no clear understanding of the pathways involved in HCM development since the clinical expression is heterogeneous. This heterogeneity has been a major bottleneck in the development of specific treatments for HCM, with clinicians opting to use proven non-specific drugs that alleviate the symptoms alone.

The growing body of research on this disease shows that there may not be a single pathway, but rather multiple different ways in which HCM mutations lead to hypertrophy. A common defining trait for these mutations is their effect on cardiac contraction rates (contractility) that are most likely caused by perturbations in calcium handling and cross-bridge regulation. The goal of this thesis research was to determine the effects of ACTC variants found in early-onset HCM by studying their effects on regulation. The Dawson lab has classified several different ACTC variants with the goal of understanding how cardiomyopathies occur and they are particularly interested in the role of ACTC mutations found in hypertrophic and dilated cardiomyopathy. Studying the effects of these mutations in vitro will allow us to determine the link between the site of
mutations and their effects on regulation, providing a model for the initial changes that lead to hypertrophy. Understanding the link between mutations and hypertrophy may allow researchers to develop a more targeted approach to treating hypertrophic cardiomyopathy. This research is especially important for young children suffering from early-onset HCM, as they cannot be treated with the same medications as adults.

1.1 Impact of Cardiovascular Disease

Cardiovascular diseases (CVDs) are one of the top five non-communicable diseases (NCDs), affecting 1 in 12 Canadians between 2012 and 2013 alone\(^1\). The improvement in sanitation and infection control in third world countries has led to a shift in prevalence from infectious disease to NCDs such as CVDs. This shift can be attributed to the health systems within these regions specializing in infection control rather than long term care of chronic CVDs\(^2\). This rise in CVDs could have a global impact as developing countries account for 97 percent of population growth worldwide\(^3\).

CVDs are also on the rise in North America, with 4.5 million patients hospitalized in the US every year\(^4\). The death toll of these diseases is approximately 23.5% in the US as most of these diseases have no cure\(^5\). The Canadian healthcare system is also greatly affected, with CVDs accounting for 16% of all hospitalizations\(^6\). Due to the chronic nature of CVDs, patients are often hospitalized multiple times, incurring an added cost of $22 billion in hospitalization fees\(^6\). This trend shows no signs of slowing over time as the risk factors associated with this disease are on a steady rise\(^6\). This trend is further compounded by the limited treatments available for CVDs as clinicians treat the symptoms rather than the underlying cause of disease.
The most severe forms of CVD may lead to sudden cardiac death (SCD), a condition where the severe dysregulation of the heart rate leads to potentially fatal arrhythmia that causes sudden loss of consciousness and potentially death. SCD may occur due to an underlying inherited CVD capable of affecting the contractility of the cardiac muscle. Cardiomyopathies (CM) are a prime example of an inherited CVD, and they account for approximately 10-15% of SCD in Western countries and an astounding 30-35% in Japan. Studying CM is therefore important since there is still no cure for this disease, and the current treatments vary greatly in efficacy.

1.2 Hypertrophic Cardiomyopathy

CMs are a group of diseases that are characterized by structural or functional abnormalities within the myocardia. CMs are the most commonly inherited CVDs and they have been categorized into four groups: restrictive, arrhythmogenic right ventricular, dilated and hypertrophic cardiomyopathy. The most prevalent forms of CM are dilated (DCM) and hypertrophic cardiomyopathy (HCM).

HCM is an autosomal dominant disorder characterized by left ventricular hypertrophy without the presence of an underlying metabolic or cardiovascular pathology. This disease is often misdiagnosed as a secondary characteristic of aortic or metabolic diseases, which cause ventricular thickening by increasing the external load on the heart. Other challenges with diagnosing this disease stem from the variability in its clinical expression. Patients could remain asymptomatic until the later stages of their life, or they may have a considerable amount of left ventricular hypertrophy before they reach adolescence.
Symptoms associated with this disease include dyspnea, angina, palpitations, dizziness, syncope and fatigue\textsuperscript{11}. HCM can also cause some patients to experience cardiac arrhythmias, as well as atrial fibrillation and ventricular tachycardia\textsuperscript{10}. These symptoms often occur because of left ventricular outflow tract obstruction (LVOTO) and mitral regurgitation due to progressive hypertrophy near the interventricular septum. This impedes the systemic circulation of blood during systole, and it may lead to arrhythmia and heart failure\textsuperscript{12}. 

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image_a.png}
\caption{A)}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{image_b.png}
\caption{B)}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{image_c.png}
\caption{C)}
\end{figure}
Patients with LVOTO have a poor prognosis and they often require some form of surgical intervention to remove the blockage. There are variations in the site of hypertrophy within the heart, resulting in a subset of patients developing this condition. For example, some individuals with apical hypertrophy remain asymptomatic for a long time, only developing cardiac arrhythmia later in life.

Differences in the site and severity of hypertrophy play a key role in the clinical manifestation of this disease as well as the age of onset. These differences make the diagnosis of HCM difficult, requiring multiple tests before it is properly diagnosed. The clinical standard for accessing cardiovascular dysfunction is the electrocardiogram (ECG), which is used to detect the electrical impulses generated by the heart. Patients with HCM have an aberrant Q wave as well as defects with the repolarization of the heart. An irregular Q-wave indicates that there are issues with the depolarization of the interventricular septum, which is commonly the site of hypertrophy. Anomalies in repolarization are a strong indicator of issues with diastole, affecting cardiac filling. These ECG abnormalities are also present in hypertensive disease, making it important to confirm the correct diagnosis.

Magnetic resonance imaging (MRI) is performed to directly measure the increase in cardiac wall thickness, with a thickness of 15 mm or greater confirming that patients
have hypertrophy\textsuperscript{16}. Taken together, ECG and MRI provide an effective way for clinicians to
detect cardiac hypertrophy but do little to define its underlying cause. The only way to
definitively find the underlying cause of HCM is to genetically test the individual. Genetic
screening has become ubiquitous in the diagnosis of HCM, since ventricular hypertrophy may be
caused by secondary diseases such as metabolic disorders (e.g. Noonan’s syndrome or Pompe’s
disease) that have a completely different pathway for causing cardiac hypertrophy\textsuperscript{17}.

1.3 Treatments Used for HCM

The genetic and physiological changes associated with HCM have been determined, but
how these changes lead to the pathophysiological condition has yet to be determined. Improving
the diagnosis does not resolve the fact that certain individuals develop \textit{de novo} HCM mutations,
making it difficult to determine the underlying cause of this disease\textsuperscript{18}. Even if this disease is
detected in its early stages, the options for treating the cardiac hypertrophy are limited.

The symptoms experienced in HCM occur due to an increase in the cardiac wall stress
which occurs as a result of impaired left ventricular relaxation leading to a build-up of diastolic
pressure\textsuperscript{19}. These symptoms are often treated with β-blockers, which are used for both
obstructive and non-obstructive forms of this disease (Figure 1B)\textsuperscript{20}. β-Blockers can reduce the
rate of cardiac contraction as well as improve ventricular relaxation. They do so by blocking β-
adrenergic receptors from binding epinephrine and norepinephrine, which are capable of
increasing the rate and force of the cardiac contraction\textsuperscript{20}. By reducing the force and frequency of
cardiac contractions, β-blockers can reduce the build-up of diastolic pressure within the heart
subsequently decreasing cardiac wall stress. These may reduce the symptoms linked to HCM,
but their efficacy for reducing cardiac hypertrophy is yet to be determined\textsuperscript{8}. 
Calcium channel blockers are often used in patients that are intolerant to β-blockers, as they are known to have the same contractile effects on the heart. These drugs have to be used with caution for HCM patients with LVOTO, or they may exacerbate the occurrence of cardiac arrhythmia. Like β-blockers, calcium channel blockers are unable to treat the underlying cause of hypertrophy, and only alleviate the associated disease symptoms.

The only proven way to reduce hypertrophy is to surgically remove hypertrophic tissue. Septal myectomies are commonly performed on patients that do not respond well to pharmacotherapy. This procedure involves the surgical removal of hypertrophic septal tissue, reducing the obstruction within the left ventricular chamber and improving the survival of patients that have obstructive HCM. There is, however, a significant risk of infection due to open heart surgery, which is why this procedure is only performed on patients with severe LVOTO.

The key to treating HCM lies in the development of therapeutics that have a specific effect on the pathway leading to hypertrophy. This approach requires knowledge of these pathways, as well as a deeper understanding of the cause of phenotypic variations associated with HCM mutations. This thesis is focused on understanding the pathway leading to the early-onset occurrence of HCM.

1.4 Inheritance of HCM

The genetic link of this autosomal dominant disease was discovered in 1989 by a group of geneticists and clinicians studying the intergenerational impact of HCM within a large family. The clinical study involved some large kindred where 20 of 24 living
members had familial hypertrophic cardiomyopathy (FHC), allowing geneticists to map the disease locus through several generations. They produced 41 polymorphic DNA probes and performed co-segregation analysis to determine the locus of the genes linked to this disease\textsuperscript{22}. The probes were used to map out which polymorphic DNA were co-inherited with HCM in the family members. This study led to the discovery of the disease gene on chromosome 14 on band 1 of the q arm (14q1), the first loci associated with HCM\textsuperscript{22}. Further molecular genetic studies led to the discovery of the following loci: 14q11, 1q32, 15q22 and 11p11, all of which encode sarcomeric proteins\textsuperscript{22–24}. The mutations were present in genes for β-myosin heavy chain (\textit{MYH7}), cardiac myosin-binding protein C (\textit{MYBPC3}), cardiac troponin T (\textit{TNNT2}), cardiac troponin I (\textit{TNNI3}), essential myosin light chain (\textit{MYL3}), regulatory myosin light chain (\textit{MYL2}), tropomyosin (\textit{TPM1}), and cardiac actin (\textit{ACTC1})\textsuperscript{4}. These sarcomeric genes became candidates for identifying and studying HCM patients.

The discovery of these genes was integral to the molecular studies of HCM, but they did not lead to the immediate discovery of a common disease pathway. Some of these mutations have varied clinical expression, making it difficult to define a clear link between disease expression and site of mutation\textsuperscript{25}. Certain sarcomere mutations, however, show a clear association between the site of mutation and the clinical expression. Mutations in \textit{MYH7} have a severe clinical expression with a significantly higher risk of SCD, while mutations in \textit{MYBPC3} tend to be relatively benign with a later-onset of symptoms\textsuperscript{25}. It is interesting to note that mutations in the \textit{MYH7} and \textit{MYBPC3} genes are the most common, as they are present in approximately 80% of all HCM cases\textsuperscript{17}. Due to their high prevalence, mutations in \textit{MYH7} and \textit{MYBPC3} have been studied extensively, allowing researchers to draw a clear link between
mutations within these genes and the resultant phenotype. These results also emphasize the importance of studying other sarcomere mutations, as they may allow researchers to identify disease pathways that have yet to be discovered.

There are hundreds of mutations present in MYH7 related to HCM and a majority of these result in amino acid substitutions in critical residues of this protein\textsuperscript{36}. These mutations are concentrated within three domains: the ATPase, actin-binding and force transmission domains. Unlike MYH7, MYBPC3 variants are not incorporated into the sarcomere, leading to defects in myosin regulation due to haploinsufficiency\textsuperscript{27,28}. Mutations in MYH7 and MYBPC3 have a similar prevalence, but their effects within the sarcomere are quite different, which further suggests that there are multiple pathways involved in the development of HCM. Given the number of mutations, studying each pathway individually and looking at the effects of each myosin regulatory protein mutation independently is a monumental task.

An alternative approach to study HCM is to observe the changes that occur with thin filament mutations. The thin filament is composed of the cardiac troponin complex, tropomyosin, and actin. These proteins have not been studied as extensively as the myosin regulatory proteins, since they are only found in 11\% of all HCM cases\textsuperscript{17}. The smaller number of HCM mutations found within these genes make it more feasible to find a link between the site of mutation and their clinical expression. Each point mutation within thin filament proteins also represent many potential disruptions in sarcomere interaction. Our research group is interested in the study of the ACTC protein, since 12 different ACTC1 mutations are implicated in the development of HCM\textsuperscript{13}. ACTC is found
at the core of the thin filament and observing the effects of ACTC1 mutations could shed light on the multiple pathways leading to HCM. My research looks at ACTC1 mutations that are involved in the development of early-onset HCM. To better explain the role of these point mutations, I will briefly go over the role of ACTC within the cardiac sarcomere.

1.5 Sarcomeric Proteins

Sarcomeres form the core contractile unit within cardiomyocytes; they are surrounded by the sarcolemma and basement membrane. The outermost basement membrane layer is composed of collagen, and it envelopes the sarcolemma to form an interface with the intracellular and extracellular environment. Transverse- (T-) tubules are interspersed between the sarcomere bundles extending the sarcolemma into the sarcomere, and bring L-type calcium ion (Ca^{2+}) channels and sarcoplasmic reticulum (SR) in close contact with the sarcomere bundles, thus playing a critical role in the excitation-contraction coupling of cardiomyocytes. This process involves the coupling of an action potential with the propagation of a depolarization wave that results in the activation of voltage-sensitive L-type Ca^{2+} channels. The influx of Ca^{2+} into the sarcomere plays a critical role in sarcomere contraction, once Ca^{2+} binds thin filament regulatory proteins.

Each sarcomere is composed of three regions: the A-band, located in the middle of the sarcomere, as well as two I-bands on either side of it (Figure 2). The A-band remains stationary, and this region includes the myosin thick filaments and cardiac myosin binding protein-C (cMyBP-C) that interacts with the myosin hinge region and actin heads. cMyBP-C acts like a “molecular brake”, slowing down actin sliding along myosin; when dephosphorylated, it plays an ancillary role in the regulation of sarcomere contraction. The I-bands are composed
of actin thin filaments that interdigitate the myosin thick filaments, along with the regulatory proteins troponin (Tn) and tropomyosin (Tm). The thick filaments can hydrolyze ATP, moving the thin filaments closer to the M-line at the center of the sarcomere. This movement leads to the shortening of the sarcomere, forming the basis for cardiac muscle contraction. The binding of myosin to actin is regulated by Tm and the Tn complex. Tm has a coiled-coil helical structure, which blocks the binding of myosin heads to actin, while the Tn complex modulates the binding of Tm to actin, allowing it to shift from a closed state to an open state upon Ca^{2+} binding.

**Figure 2. Cardiac sarcomere.** A simplified diagram of the cardiac sarcomere highlighting several important proteins and structural features.

There are two fundamental factors that affect the contractile force and activation in the cardiomyocyte: myosin and actin cross-bridge formation, and Tm and Tn
regulation. It is also clear that Ca$^{2+}$ handling has a central role in the underlying mechanism of hypertrophy since it is involved in regulating multiple interactions within the sarcomere.

1.6 Cross-Bridge Cycle

The movement of thin and thick filaments is sarcomeres producing contractile force is generated through the cross-bridge cycle. Actin thin filaments function as “tracks” for the myosin motor protein, which hydrolyzes ATP to change its conformation and generate tension against the thin filament$^{29}$. The core of the thin filament is composed of filamentous actin (F-actin), which is a helical polymer formed when the DNase I binding loop of an actin monomer inserts into the hydrophobic cleft of an adjacent monomer$^{33}$. Cardiac thick filaments are composed of myosin II, which has two catalytic heads capable of interacting with two adjacent actin subunits within F-actin$^{34}$. Each myosin protein is composed of four subdomains linked with connectors that allow them to change conformation in response to ATP and actin binding$^{34}$. The subdomains include the motor domain, the lever arm, dimerization region (coiled-coil tail) and a targeting region that keeps myosin bound to the adjacent myosin tail regions within the thick filament. The motor domain is on the 50 kDa myosin head and is the site of actin binding and ATP catalysis. The head is composed of an upper (U50) and lower (L50) domain region, responsible for binding actin; the N-terminal region of the myosin head is joined to relays and converter regions that trigger conformational changes in myosin during the cross-bridge cycle.

The cycle begins when ATP rapidly binds to the myosin head, triggering its release from the actomyosin complex. Once bound, ATP triggers a shift in the converter domain near the lever arm, changing the conformation of the myosin head into a “recovery stroke”. This allows the lever arm to reorient itself before the power stroke, allowing myosin to hydrolyze ATP into ADP.
and P_i. The next step occurs immediately after ATP hydrolysis, leading to a shift in the myosin conformation that allows the L50 and U50 regions to interact with the actin subunits. The initial binding of myosin to actin occurs at the L50 domain, where the actin-binding loops and the helix-loop-helix regions form weak hydrophobic interactions with actin subdomain 1 (SD1) and subdomain 3 (SD3). The weak electrostatic interactions stabilize myosin binding to actin and enable the closure of the cleft between the upper and lower region. This allows the movement of the U50 domain to form a strong stereospecific contact between myosin and actin.

The transition from weak to strong binding is followed by the myosin power stroke along actin, sliding the thin filaments towards the center of the M-line. As soon as the power stroke is completed, ADP and P_i are released from the nucleotide binding site, leaving myosin in a "rigor state". This marks the end of a cross-bridge cycle, shifting myosin to a state that allows it to bind ATP once more.

1.7 Tm and Tn Regulation

Actomyosin interactions are responsible for force generation within the sarcomere, but the cross-bridge cycle is not constitutively active. These interactions are allosterically regulated by Tm and Tn in response to the concentration of Ca^{2+} within the sarcomere. Tm is composed of two alpha helices forming a coiled-coil structure that wraps around the actin thin filament, blocking myosin-binding sites. Tm is held in place by the Tn complex, which is made of the three subunits TnI, TnC and TnT. TnC plays a key role in regulation, as it binds to Ca^{2+}, modulating the shift of Tm from a closed “B-state” to the intermediate “C-state”. TnI has a dual role: it anchors Tm to the thin filament, and phosphorylation of this
A subunit is able to increase the rate of Ca\textsuperscript{2+} dissociation from TnC, modulating Ca\textsuperscript{2+} sensitivity in response to adrenergic stimulus\textsuperscript{9}. Finally, the TnT subunit links the Tn complex to Tm, acting as a relay between the other two subunits.

In the presence of low Ca\textsuperscript{2+}, TnI keeps Tm anchored to actin in the B-state, blocking the binding of the myosin head\textsuperscript{35}. When the SR releases Ca\textsuperscript{2+} into the sarcomere, TnC binds Ca\textsuperscript{2+}, allowing Tm to shift from the B-state to the C-state\textsuperscript{35}. Once Tm is in the C-state, initial myosin binding takes place, further shifting Tm into the fully open M-state\textsuperscript{35}. This regulatory complex plays a key role in excitation-contraction coupling, as it controls the contractility of each sarcomere within the cardiomyocyte ensuring that this ATP-dependent process does not exert too much energy\textsuperscript{36}.

Changes in Ca\textsuperscript{2+} sensitivity have been linked to phenotypes observed in HCM, such as fatal arrhythmias and increased ventricular hypertrophy\textsuperscript{17}. A study on transgenic mice with increased myofibrillar Ca\textsuperscript{2+} sensitivity showed abnormalities in the ventricular action potential that led to high beat-to-beat variability at faster heart rates\textsuperscript{37}. These factors made the mice susceptible to fatal arrhythmias, a trait which is often found in severe cases of HCM. Increased \textit{in vitro} Ca\textsuperscript{2+} sensitivity correlates with a higher susceptibility to SCD\textsuperscript{38}. Studying changes in sarcomere Ca\textsuperscript{2+} sensitivity can help us understand the underlying mechanism responsible for the development of early-onset HCM due to point mutations in the \textit{ACTC1} gene.

1.8 \textit{ACTC1} Mutations

The best way to elucidate the underlying cause of this disease is to determine the common features present in all HCM-related mutations. While the molecular pathways have yet to be defined, the key feature of all these mutations is their effect on Ca\textsuperscript{2+} sensitivity\textsuperscript{17}. A
majority of the research has been performed on regulatory elements that directly or indirectly affect \( \text{Ca}^{2+} \) handling \((i.e., \text{cMyBP-C, TnC, TnT})\). It is interesting to note that there are sarcomeric proteins that do not directly affect \( \text{Ca}^{2+} \), but still have an overall effect on sarcomere \( \text{Ca}^{2+} \) sensitivity. A key example of this is ACTC, a protein that interacts with all major components of the sarcomere.

\textit{ACTC1} mutants have been studied extensively and are the only sarcomeric mutations involved in both HCM and DCM\(^{39}\). This situation makes ACTC variants prime candidates for biochemical studies, allowing researchers to observe how single amino acid changes lead to different pathologies. Like HCM, DCM is also caused by changes in \( \text{Ca}^{2+} \) handling within the sarcomere\(^{40}\). However, the phenotype associated with this disease is thought to occur as a result of TnI phosphorylation uncoupling rather than specific changes in \( \text{Ca}^{2+} \) sensitivity\(^{17}\). How do differences in the site of point mutations in the same gene cause two separate diseases?

The first HCM-linked mutation found through candidate genetic testing resulted in a substitution of the 101\(^{st}\) residue glutamic acid, for lysine (E99K-ACTC)\(^{41}\). Two different families in Spain had more than half of the individuals expressing this autosomal dominant mutation, and individuals with the E99K-ACTC variant had a typical clinical presentation of severe apical hypertrophy\(^{41}\). This amino acid substitution occurred within actin subdomain 1, which interacts with a positively charged lysine residue on the L50 domain of myosin\(^{42}\). This charge reversal can destabilize the strong binding of myosin with actin, as transient binding is a key step in the conformational change in the U50 and L50 domain\(^{35}\). \textit{In vitro} studies of this variant showed a decrease in filament sliding velocity, which is indicative of issues with direct actomyosin
interactions\textsuperscript{13}. E99K-ACTC obtained from patients hearts was studied together with Tm, Tn and other sarcomeric proteins within reconstituted myofilaments; these myofilaments had impaired relaxation, as well as higher tension at low Ca\textsuperscript{2+}, which are key factors that lead to hypercontractility\textsuperscript{43}. Studies with transgenic mice further confirmed the link between severe apical hypertrophy and the E99K mutation\textsuperscript{44}.

These results are interesting since changes in the E99 residue of actin should not have a direct effect on Ca\textsuperscript{2+} handling. This change in Ca\textsuperscript{2+} handling suggests that these point mutations may affect cooperative binding of other thin filament proteins, rather than destabilizing actomyosin interactions alone. It is important to study ACTC variants within the myosin binding subdomain in the context of regulated thin-filaments, so that we may understand the possible changes on Ca\textsuperscript{2+} handling (Figure 3A).
F90Δ, H88Y and R95C have been defined as M-class mutations due to their proximity to the myosin binding site on actin\textsuperscript{13}. All three variants interact with loop 3 of myosin, making them prime candidates for altering actomyosin interactions. Actin and myosin interactions can be observed \textit{in vitro} by directly measuring changes in actin sliding velocity along myosin in the absence of regulatory proteins\textsuperscript{45}. While R95C and H88Y show no changes in \textit{in vitro} sliding velocity, F90Δ showed an increase in sliding velocity relative to recombinant human ACTC (WTrec)\textsuperscript{46}. These inconsistencies show that the underlying disease mechanism is most likely related to the disruption of thin filament regulation rather than specific actomyosin interactions alone\textsuperscript{44}.

It is clear that further experimentation on M-class variants must to be carried out with regulatory proteins\textsuperscript{39}. I will be studying the regulatory thin filament complexes composed of F90Δ-ACTC and H88Y-ACTC, both discovered in preadolescent patients.

1.8.1 F90Δ-ACTC

F90Δ-ACTC was identified through the clinical testing of children under the age of 13, with 7 of 42 of them being diagnosed with HCM within a year of their birth\textsuperscript{47}. Approximately 5\% of the 79 patients had the F90Δ mutation, which is about 4 patients out of the entire cohort\textsuperscript{47}. This was a unique study, as clinicians did not attribute the development of ventricular hypertrophy to inherited mutations within young children due
to a lack of understanding of how ventricular hypertrophy progressed\textsuperscript{47}. Past studies attributed rapid changes in ventricular wall thickness in adolescents to growth spurts\textsuperscript{48}. This idea was disproven by Kaski \textit{et al.} in 2009 who showed a link between the presence of sarcomere mutations in preadolescent children and HCM, further proving that this disease is first and foremost a disease of the sarcomere. This group genetically tested young patients and kindred with HCM, where they found the link between left ventricular hypertrophy sarcomere mutations, rather than neuromuscular, metabolic or syndromic disorders\textsuperscript{47}.

1.8.2 H88Y-ACTC

Shortly after the discovery of F90Δ, H88Y-ACTC was discovered by a group of scientists and clinicians lead by Dr. Morita at Harvard Medical School, who conducted a genetic study on a family where 84 children had been diagnosed with idiopathic HCM\textsuperscript{49}. This group of researchers endeavored to find mutations linked to sporadically-occurring HCM. By sequencing a group of candidate genes, they located a missense mutation encoding a change at the 88\textsuperscript{th} amino acid of ACTC, where a histidine was replaced with tyrosine\textsuperscript{49}. The proximity of F90Δ-ACTC and H88Y-ACTC, as well as their occurrence in pre-adolescent children could show a link between the site of these mutations and a pathway that may be involved in the early-onset development of HCM. It is also interesting to note that these variants have not been studied by other labs, presumably due to their lower prevalence, as well as the difficulty in producing recombinant ACTC.
1.9 Testing Sarcomeric Variants

Cardiac remodelling is linked to changes in protein-protein interactions due to sarcomere mutations. Hypertrophy often occurs due to a compensatory mechanism, which leads to myocyte enlargement. This progressive enlargement leads to biomechanical stress that progressively deteriorates cardiac function. The first step in defining the pathways responsible for this compensatory hypertrophy is to determine how HCM mutations alter regular sarcomere function. One way to study changes in the sarcomere is to measure changes in cross-bridge activation.

The force generated within the sarcomere is proportional to the rate of myosin and actin cross-bridge formation. Measuring changes in the rate of ATP turnover can be used to determine the effects of each variant on contractile force. An enzymatic assay such as the actin-activated myosin ATPase assay may be used to quantify the amount of Pi released once each variant actin filament has interacted with myosin. The rate of Pi production from this reaction has been used to study the kinetics of each ACTC variant.

This assay has been performed on F90Δ and H88Y, and the resulting ATPase activity of each variant was plotted against their concentration, then fit to a Michaelis-Menten curve. H88Y showed no significant changes in its k_cat relative to WTrec ACTC, while F90Δ had a lower k_cat showing that this mutation could adversely impact actomyosin interactions. These findings did not provide insight into the link between early-onset HCM and the effects of these mutations. The actomyosin interactions were not severely affected, and the system did not contain the key regulatory proteins Tm and Tn. Bai et al. studied reconstituted fibres containing the ACTC variants E99K and
A230V, in both the presence and absence of Tm and Tn. They demonstrated that the regulatory proteins acted as a fail-safe mechanism by exerting a positive allosteric effect on the reconstituted filaments.

Dahari et al. further emphasized that the changes observed in direct actomyosin interactions were often too severe, and that Tm and Tn compensate for these changes. These regulatory proteins play a role in the binding topology of actin and myosin, which further shows that regulatory proteins have to be considered in the study of ACTC variants.

Actomyosin regulation has been studied using regulated thin filaments (RTFs) of recombinant actin complexed with tissue purified Tn and Tm. These RTFs are used in the myosin ATPase assay and the in vitro motility assay (IVM), which have been modified to study the rates of RTF activation at different Ca$^{2+}$ concentrations.

1.10 Previous Research

The prevailing hypothesis within the field is that mutations involved in HCM cause an increase in sensitivity of sarcomeres to Ca$^{2+}$ that subsequently causes left ventricular hypertrophy. A review by Dr. Marston lists 71 independent measures of Ca$^{2+}$ sensitivity for sarcomere mutations implicated in HCM. Regardless of where these mutations occur, every HCM-linked mutation causes an increase in Ca$^{2+}$ sensitivity. This trend has been further emphasized by other researchers within the field, who have proposed a mechanism for the subsequent progression of hypertrophy within HCM patients. These reviews do not look at specific pathways involved in the occurrence of this disease, but instead define a common underlying mechanism that could lead to hypertrophy. My study looks at the role of ACTC
mutations involved in the development of early-onset HCM, so that we can understand which pathways lead to the rapid occurrence of this disease. It may be ineffective to treat patients with early-onset hypertrophy the same way as older patients with HCM. Studying the pathway leading to early-onset hypertrophy could lead to the development of better therapeutics for young patients suffering from this disease.

1.11 Hypothesis

My research is aimed at determining the role of early-onset ACTC variants in the development of HCM. I hypothesize that F90Δ and H88Y will cause an increase in thin-filament Ca$^{2+}$ sensitivity, as well as an increase in \textit{in vitro} velocity during the cross-bridge cycle. I will compare the shift in Ca$^{2+}$ sensitivity and velocity for both variants to determine whether the proximity of these variants influences thin-filament regulation.

1.12 Research Aims

I expect to see an increase in the Ca$^{2+}$ sensitivity of the early-onset ACTC variants relative to WTrec RTFs. The severity and nature of Ca$^{2+}$ sensitivity will also be assessed in each experiment, allowing us to observe how the location of each mutation could play a role in disrupting the Tm and Tn interactions within the thin filament. I aim to characterize the specific effects of each variant, showing that all HCM mutations, even those not directly involved in Ca$^{2+}$ handling, have an influence on Tm and Tn. These changes subsequently lead to the increased Ca$^{2+}$ sensitivity causing ventricular hypertrophy.

Aim 1. Purify the proteins involved in the production of RTFs
a) Purification of recombinant F90Δ and H88Y produced with a baculovirus expression system
b) Purification of Tn and Tm proteins from fresh bovine tissue

Aim 2. Determine the Ca\(^{2+}\) sensitivity of the RTFs. F90Δ and H88Y ACTC RTFs pCa\(_{50}\) values will be compared with WTrec ACTC RTFs

a) The IVM assay will be used to observe changes in thin filament activation at different pCa values
b) The RTF ATPase assay will be used to determine the RTF activation at different pCa

1.13 Summary

The goal of this thesis research is to determine the effects of ACTC variants found in early-onset HCM by studying their effect on regulation. Due to their low prevalence, F90Δ-ACTC and H88Y-ACTC and have not been studied as extensively as the E99K-ACTC. This has led to a gap in understanding the specific pathways that may lead to the development of early-onset HCM, since E99K-ACTC is generally found in elderly patients. Understanding the involvement of these ACTC variants in the disruption of Tm and Tn interactions will lead to greater insight on rapid hypertrophy without the presence of prolonged cardiac wall stress. This will lead to a more targeted approach in treating early-onset HCM, rather than utilizing therapeutics targeting pathways involved in the late-onset form of HCM.
2. Materials and methods

2.1 Reagents

The reagents used in this study were purchased from Fisher Scientific (Markham, ON) unless otherwise stated. The SDS-PAGE gels were made using 29:1 bisacrylamide solution from Bio-Rad (Hercules, CA). The reagents used for insect cell culture, such as the Fetal Bovine Serum (FBS) and Penicillin/Streptomycin (Penstrep), were purchased from Gibco (Life Technologies, Mississauga, ON). Supplemented Grace’s Insect Medium was purchased from Wisent Bio Products (Saint-Jean-Baptiste, QC). The HiTrap DEAE fast flow, Mono Q 10/10 and Q Sepharose fast flow columns were all purchased from GE Lifesciences (Piscataway, NJ). The Ni\(^{2+}\)-NTA superflo flow affinity columns were purchased from Qiagen (Valencia, CA).

2.2 Production of Recombinant ACTC

2.2.1 Maintenance of Insect Cell Culture

A continuous suspension culture of Spodoptera frugiperda (Sf21) insect cells was maintained in a 27 °C incubator. These cells were used to seed new suspension cultures, which were infected with the baculovirus used to produce recombinant ACTC. The Sf21 continuous culture was split after 3 days once it reached a density of 1.2x10\(^6\) cells per mL. On the third day, the culture was split into a 500 mL flask, and fresh Grace’s Insect Medium containing FBS and Penstrep were added to top up the culture to 500 mL.
2.2.2 Cloning F90Δ and H88Y ACTC variants

Site directed mutagenesis was carried out by Mutagenex (Suwanee, GA) on pAcUW2Bmod-ACTC (WTrec) transfer vector to produce ACTC mutants (H88Y and F90Δ). These constructs were verified by the manufacturer and the University of Guelph’s Lab Services. This verification process was carried out by previous graduate student Liu (2016). Once the constructs were verified, they were co-transfected with Bsu26I-linearized BacPAK6 baculovirus DNA into Sf21 cells. The amplification and verification of these viruses was also carried out by Liu (2016) as described.

2.2.3 Generation of Recombinant ACTC Using Baculoviruses

The recombinant ACTC constructs obtained from Mutagenex were previously co-transfected using the Sf21/baculovirus system protocol of Yates, Otley and Dawson (2007). Once the cells were transfected, each baculovirus stock was amplified, titred and stored at 4 °C covered in aluminum foil to reduce light exposure. The amplified stocks were then used to infect a 500 mL culture of Sf21 cells (from suspension culture) once the cells had reached a density of 1x10^6 cells/mL. The volume of virus used to infect the culture was based on the virus titre, ensuring a multiplicity of infection (MOI) of 1. The infected cell culture was incubated at 27 °C, and then harvested at 96 hrs post infection (hpi). Cell pellets obtained from the infected suspension culture were flash frozen in liquid nitrogen and stored in a -80 °C freezer.
2.3 Protein Purification

2.3.1 Purification of His-Tagged G4-6

Gelsolin is an actin capping and severing protein with a high affinity for binding actin in the presence of Ca$^{2+}$. Gelsolin’s domains 4 to 6 (G4-6), near the C-terminus, allow it to bind to actin, while the domains near the N-terminus are responsible for severing actin polymers. The protocol for the purification of actin proteins is based on an affinity chromatography method developed by Ohki et al. (2009), which utilizes the selective binding properties of His-tagged G4-6 for actin present within the harvested cell lysate.

The His-G4-6 was expressed in Escherichia coli using a cold induction protocol adapted by Ms. Ojehomon. Once induced, the cell pellet containing the His-G4-6 was lysed using a mechanical French press. The lysate was then cleared by centrifugation at 1,000 x g for 20 min at 4 °C, and the supernatant containing soluble His-tagged G4-6 was loaded onto a 5 mL Ni-NTA column (Qiagen). The Ni-NTA column was then attached to an AKTA FLPC (GE Amersham), and the column was washed with 30 mL of binding buffer (10 mM Tris.HCl, pH 8.0, 10 mM imidazole, 50 mM KCl, 5 mM CaCl, 1 mM PMSF) followed by the protein elution with a KCl gradient from 0 to 100% of 50 mM KCl at a flow rate of 3 mL/min for 10 minutes collecting a total volume of 30 mL. One mL fractions were collected and the peak fractions were dialyzed in G-buffer (2 mM Tris.HCl, pH 8.0, 0.2 mM CaCl, 0.2 mM ATP, 0.5 mM beta-mercaptoethanol and 0.002% NaN$_3$), flash frozen in liquid nitrogen and stored at -80 °C.
2.3.2 Myosin Purification From Rabbit Soleus Muscle

Full length myosin was purified from the soleus muscle of freshly slaughtered rabbits. This tissue contains approximately 90% type I myosin (low ATPase activity) and 10% type II myosin (high ATPase activity), and is comparable to human ventricular myosin, which is 10% alpha-myosin heavy chain (high ATPase activity) and 90% beta-myosin heavy chain. Rabbit soleus is a good replacement for human tissue purified myosin, since it is easier to acquire, and should have similar results in enzymatic assays.

Myosin was purified according to a protocol adapted from Margossian and Lowey (1982). The myosin purification was started immediately after the rabbit soleus was obtained from the abattoir (Abate Rabbit Packers Ltd., Arthur, Ontario). The following procedures were performed on site at the abattoir since myosin protein can degrade quickly post-mortem. The soleus muscle was excised from the carcass and ground. The ground muscle was immediately added to an extraction solution containing 0.3 M KCl, 0.15 M potassium phosphate (pH 6.5), 20 mM EDTA, 5 mM MgCl₂, and 1 mM ATP. The extraction was performed in a graduated cylinder, which was inverted and mixed by hand for 10 min until cold milliQ water was added to dilute this mixture 4-fold. This dilution stopped the extraction process and the remaining purification steps were performed in the Dawson lab. The extraction mixture was left to settle into distinct phases with the myosin being present in the precipitate. This precipitate was pelleted out by centrifugation at 12,000 x g and dialyzed into 50% glycerol, and the samples of full length myosin were flash frozen and stored at -80°C.
2.3.3 Heavy Meromyosin Preparation

Heavy Meromyosin (HMM) contains the two catalytic myosin heads along with a small portion of the tail region. HMM is used in place of full-length myosin as it retains its ATPase activity while remaining soluble. HMM was produced by cleaving full-length myosin at the α-chymotrypsin site, which separates a portion of the myosin tail region (LMM) that has a coiled-coil motif. This protocol was described by Margossian and Lowey (1982) and it was modified to improve the yield and purity of the HMM produced. Full-length myosin (in 50 % glycerol) was dialyzed into 0.1 M phosphate buffer (pH 6.5). The dialyzed full-length myosin was digested using 0.2 % α-chymotrypsin solution (2 mg/mL) for 10 mins, and the digestion was stopped using 0.3 mM PMSF. This solution was then dialyzed in the phosphate buffer containing 0.5 mM PMSF to ensure that the reaction had stopped completely, reducing any further breakdown of HMM due to residual amounts of active α-chymotrypsin. The dialysate containing the cleaved myosin was then centrifuged at 100,000 x g at 4 °C for 12 min. The supernatant containing HMM was obtained and stored at -80 °C and the pellet was discarded. An aliquot of HMM was thawed for each experiment and dialyzed in 1xABB buffer (20 mM imidazole (pH 8), 10 mM KCl, 2mM MgCl₂, 1 mM DTT, 1 mM EGTA ) without ATP, to reduce any inorganic phosphate (Pᵢ) present within the frozen HMM aliquots, since the Pᵢ would give a false positive for the RTF ATPase assay.
2.3.4 ACTC Purification

The ACTC purification protocol was adapted from the cytoplasmic actin purification method developed by Ohki et al. (2009). Briefly, this method utilizes His-tagged G4-6 (described in section 2.2.1), which binds to recombinant ACTC produced by the Sf21/baculovirus system (section 2.1.3), and then purified using affinity chromatography (Figure 4). Approximately 40 mL of lysis buffer (10 mM Tris (pH 8.0) 5 mM CaCl₂, 0.2 mM PMSF, 0.01 mg/mL leupeptin, 1 mM ATP, 2 mM β-mercaptoethanol) was added to thawed (on ice) harvested ACTC cell pellets (section 2.1.3). Glass beads were added to the cell pellet which was then vortexed at 1-minute intervals, ensuring that the cell pellet was kept on ice for 3 min between each interval. This step was repeated six times, mechanically lysing the cells and releasing recombinant ACTC protein. Fifty mM KCl and 10 mg of His-G4-6 were added to the cell pellet and left overnight at 4 °C on a rotator for mixing. The lysed cell pellet was centrifuged the next day at 50,000 x g for 30 min. A 5 mL Ni²⁺-NTA column was washed with milliQ water, then equilibrated with binding buffer (10 mM Tris (pH 8.0) 50 mM KCl, 5 mM CaCl₂, 0.2 mM PMSF, 1 mM ATP, 2 mM β-mercaptoethanol) while the lysed cell pellets were being centrifuged at 50,000 x g. The supernatant was obtained from the centrifuged ACTC cells pellets and circulated through the Ni-NTA column for 90 mins. The column was washed with 30 mL binding buffer, followed by a second wash with 40 mL binding buffer containing 20 mM imidazole to remove non-specifically bound protein. After the second wash, an equilibrated DEAE column was attached to the Ni-NTA column for the third and final wash with ACTC elution buffer (binding buffer with 0.2 mM EGTA and 0.5 mM MgCl₂). The EGTA chelates the calcium within the column, releasing ACTC from the His-G4-6, since Ca²⁺ is required by His-
G4-6 to bind ACTC. Once the ACTC eluted onto the DEAE column, the column was removed and G4-6 elution buffer (binding buffer with 0.2M of imidazole) was applied to the Ni-NTA, eluting eight 1.5 mL fractions. Finally, the ACTC was recovered from the DEAE column using a high KCl (120 mM) buffer, eluting eight 0.5 mL fractions. Bradford assay dye was used to determine the ACTC protein-containing fractions, which were pooled and dialyzed in G-buffer overnight (0.2 mM CaCl₂, 0.2 mM ATP, 0.5 mM beta-mercaptoethanol, 0.002% NaN₃). Samples were either used to produce RTFs immediately, or flash frozen in liquid nitrogen and stored at -80 °C.

2.3.5 Bovine Cardiac Ether Powder

The tissue-purified regulatory proteins Tm and Tn were purified using a bovine ventricular cardiac (BVC) ether powder. This powder is a preserved form of the bovine ventricular tissue, which can be stored in -80 °C for several years and thawed prior to
Figure 4. Progress of ACTC purification. Each number (i to vi) represents a wash or elution step. (i) The harvested cell pellet obtained from the infected Sf21 cell culture containing recombinant ACTC. (ii) The cell pellet was lysed and His-G4-6 added to the mixture to bind ACTC. (iii) The lysed cell pellet was bound to the Ni-NTA column and washed with binding buffer and binding buffer with imidazole to remove any loosely bound protein. (iv) A DEAE column was connected to the Ni-NTA column and these were washed with ACTC elution buffer. All the ACTC eluted onto the DEAE column. (v) ACTC eluted from the DEAE column and (vi) G4-6 eluted from the Ni-NTA column.

purification. BVC ether powder was produced from the hearts of freshly slaughtered cows which were obtained from the Department of Animal Biosciences (University of Guelph). The protocol
for producing this powder was described by Tobacman and Adelstein (1986) based on that of Greaser and Gergely (1971). The left ventricular tissue of the bovine heart was weighed and ground. Ground tissue was then blended in a solution containing 0.5 M boric acid, 50 mM sodium borate, (pH 4.75) 2 mM DTT, and 0.125 M KCl, and 0.2% Triton X-100 until homogeneous. This mixture was washed in this buffer four times, with the final wash containing 95 % ethanol. After this wash was completed, the mixture was centrifuged at 4,000 x g and the pellets were combined, wrapped in cheese cloth, dipped in anhydrous ether, and dried overnight. The dried powder was crumbled, added to 50 mL conical tubes, and stored at -80 °C for future purifications.

2.3.5 Troponin Purification

Tn and Tm are purified from BVC ether powder (described in section 2.3.5). The BVC ether powder was brought to room temperature and added to an extraction buffer (10 mM Tris.HCl(pH 8.0) 1 mM DTT, 1 M KCl, 0.04% NaN₃, 5 mg/mL TPCK, 5 mg/mL TLCK), which was left on a stir plate at 4 °C overnight. After the extraction was complete, the mixture was centrifuged at 18,000 x g for 30 min, and the supernatant was obtained. A series of ammonium sulphate precipitations were performed with the supernatant, gradually adding ammonium sulphate powder to bring the solution from 0 to 30 % parts per thousand (ppt) of ammonium sulphate. Once the solution was at 30 % ppt ammonium sulphate, it was centrifuged at 8,000 x g for 15 min, and the supernatant decanted. More ammonium sulphate was then added to this supernatant until this solution had 45 % ppt ammonium sulphate. This mixture was centrifuged as before, and the pellet was resuspended in a homogenizing buffer (10 mM Tris.HCl (pH 8.0) 1 mM DTT, 0.01%
NaN₃, 5 mg/L of TPCK, 5 mg/L of TLCK, 0.3 mM PMSF), while the supernatant was stored for Tm purification. The resuspended pellet was dialyzed (10 mM Tris.HCl (pH 8.0) 1 mM DTT, 0.01% NaN₃, 5 mg/L of TPCK, 5 mg/L of TLCK, 0.3 mM PMSF) at 4 °C overnight. The dialysate was cleared by centrifugation at 18,000 x g for 15 min, and loaded onto a 20 mL MonoQ 10/10 fast flow column (GE Amersham). The loaded column was washed with 0.14 M NaCl buffer, followed by a gradient wash from 0.14 to 0.6 M NaCl when the eluate was collected. The chromatogram was used to determine peak fractions, which were analyzed on a 10 % SDS PAGE gel to determine if the peak samples were pure (TnI is 23.9 kDa, TnC is 18 kDa and TnT is 34 kDa). The protein-containing fractions were then pooled, dialyzed in 2 L of Tn dialysis buffer (10 mM Tris.HCl (pH 8.0) 2 mM DTT and 0.01% NaN₃) and flash frozen for storage at -80 °C.

2.3.6 Tropomyosin Purification

The stored supernatant from the 45 % ammonium sulphate fraction was further saturated with ammonium sulphate until the solution was 65 % ammonium sulphate. The sample was centrifuged for 5,000 x g for 15 min. The pellet was then resuspended with homogenizing buffer and 1 M NaOH was added to raise the pH to 8.0 prior to overnight dialysis at 4 °C in Tm dialysis buffer A (50 mM Tris.HCl (pH 8.0) 0.01% NaN₃, 1 mM DTT, 5 mg/L TPCK, 5 mg/L TLCK, 5 M urea). The dialysate was then centrifuged at 3,000 x g for 15 min, and the supernatant was loaded onto a 25 mL Q-Sepharose fast flow anion-exchange column. The protein was eluted with a gradient from 0 to 0.6 M NaCl in 1 ml fractions. The peak fractions were selected using the chromatogram and analyzed on a 10 % SDS PAGE gel to determine sample purity (Tm is 35
kDa). The protein-containing fractions were dialyzed overnight Tm dialysis buffer B (10 mM Tris.HCl (pH 8.0), 2 mM DTT, 0.01% NaN₃), flash frozen and stored at -80 °C.

2.4 Assays

2.4.1 RTF Production

Regulated thin filaments (RTFs) are a complex between Tn, Tm and recombinantly produced ACTC proteins. The thin filament proteins were added to the dialysis tubing directly at a molar ratio of 7:3:3 (22.2 μM ACTC, and 9.43 μM Tm and Tn). The proteins were then dialyzed for 2 days in 500 mL of ABB, changing the buffer once after dialyzing overnight, and then again approximately 7 to 8 hours after the previous buffer change. Once dialysis was complete, the RTFs were removed from the dialysis tubing and used directly for the RTF ATPase assay.

2.4.2 RTF ATPase assay

The actin-activated ATPase assay employed is an established assay used to characterize the enzymatic activity of myosin in the presence of actin. Myosin activity is used as an indirect measure of functional changes that may occur in actin variants. This assay quantifies the ATP turnover due to the activation of myosin ATPase during its interaction with actin. The quantification is colorimetric as an ammonium molybdate and ferrous sulphate solution reacts with Pᵢ produced during the hydrolysis of ATP producing a blue color. An increase in Pᵢ formation indicates that there is an increase in actin-
activated myosin ATPase activity. The intensity of this blue color is used to determine the level of myosin activation.

The RTF ATPase assay is a modified version of the actin-activated ATPase assay, which allows the measurement of changes in myosin activation in response to different levels of regulation. The regulation of RTFs can be modulated using buffers with different Ca$^{2+}$ concentrations, or pCa (-log of Ca$^{2+}$) buffers. There are 11 pCa buffers: pCa 10, 8, 7.5, 7.3, 7, 6.8, 6.5, 6.0, 5.5, 5.0 and 4.5. The activation of the RTFs at different pCa buffers was used to plot a pCa curve and comparing the activation of each variant RTF at half maximal activation (pCa$_{50}$) can report on changes in F90Δ and H88Y RTFs relative to WTrec RTFs.

This assay was performed on a new 96 well plate each time. The pCa buffers were made first, and the method for producing these buffers slightly modified from the previous protocol used by Liu (2016)$^{51}$. Instead of freshly preparing each 20X pCa buffer for every experiment, stocks of activating (pCa 4.5) and relaxing solution (pCa 10) were made in advance and stored at -20 °C. An aliquot of each of these buffers was thawed on the day of the RTF ATPase assay, and these buffers were mixed in different proportions to make all 11 of the 100 μL 20X pCa buffers. It is important to note that the final composition of the pCa buffers were not changed. This method ensures that stocks of the activating and relaxing solutions can be made at once and stored together (without ATP), reducing the variability of pCa buffers between each assay. Ten μL of 20X pCa buffer was then mixed with 88 μL of RTFs in a 600 μL microfuge tube. Phosphate standards are added to the 96 well plates first, and they were aliquoted in triplicate into the bottom 2 rows of wells in the plate. Once the phosphate standards were added, the dialyzed HMM was diluted to 0.25 mg/mL in ABB without ATP. Before each sample was
aliquoted into the 96 well plate, 2 µL of 1 mM ATP was added to the microfuge tubes containing RTFs and pCa buffers.

Once the ATP was added to the RTFs with pCa, 25 µL of the samples were added to the 96 well plate in triplicate. This is followed by the immediate addition on 25 µL of HMM into all wells, excluding the control and phosphate standard wells. The reaction was left to incubate for 40 min, covered with another 96 well plate that is enveloped in aluminum foil. Reactions were stopped with the addition of 50 µL of the “stop” solution containing SDS and EDTA. Two hundred µL of coloring solution (0.5% ferrous sulfate, 25% of 2% ammonium molybdate, 4 N H₂SO₄) was added to the mix. The reduction of ferrous sulphate by phosphomolybdate produces a blue color, and the absorbance was read at 750 nm.

The absorbance values of the phosphate standards were used to generate a standard curve as each of their absorbance values is used to extrapolate the Pᵢ concentrations of the RTF and pCa samples measured from the 96-well plate. This concentration is then divided by the total time of the reaction to obtain the myosin activation rate in µM/min. This rate was calculated in an Excel spreadsheet and these data were plotted using Graph Pad Prism 5 (San Diego, California) against the pCa value of each well. A four-parameter log(agonist) vs. response fit was used to generate the pCa curve. The pCa at half-maximal activation is the pCa₅₀ value, and this was used as a metric for determining the shift in Ca²⁺ sensitivity relative to WTrec RTFs.
2.5 IVM assay

The *in vitro* motility assay (IVM) utilizes fluorescence microscopy to visualize RTF movement along HMM that is bound to a nitrocellulose coverslip. This is a well-established assay within the field and the current protocol has been adapted from the work of Toyoshima et al. (1987). The buffers and proteins are added stepwise to a flow cell (Figure 5A), which is constructed by taping a nitrocellulose coverslip to a microscope slide. The double-sided tape is attached at the sides of the coverslip producing a channel at the center of the coverslip which allows buffer and protein samples to be flowed through.

The accuracy of this experiment is highly dependent on the quality of HMM bound to the nitrocellulose coverslip. Inactive HMM heads cannot hydrolyze ATP nor catalyze the movement of RTFs, creating a false negative. The probability of these false negatives can be decreased significantly by pelleting out inactive or “dead” HMM heads using a technique known as deadheading. Deadheading was started by first binding HMM to F-actin over a 40-min period, ensuring no premature release of F-actin from HMM due to residual ATP contamination. Once bound, the mixture was centrifuged at 375,000 x g for 15 min. This centrifugation sediments all HMM bound to F-actin, regardless of its activity. This pellet was then resuspended in 200 μL of 1xAB (25 mM KCl, 25 mM imidazole, pH 7.5, 10 mM DTT, 4 mM MgCl₂, 1 mM EGTA), and 0.1 M ATP was added. Immediately after the addition of ATP, the resuspended pellet was centrifuged at 375,000 x g for 15 min. The addition of ATP to the resuspended pellet releases active HMM which sediments at 375,000 x g. Thus, the supernatant contained active HMM, and the pellet contained dead HMM. Once the supernatant was collected, 0.1 M ATP was added again and centrifuged further as before to ensure that any residual inactive HMM and F-actin
were removed. This step was repeated three times, and the supernatant obtained from the final centrifugation was analysed by SDS PAGE using a 10% acrylamide gel. The ideal sample of deadheaded HMM should not have any actin contamination present (Figure 5B). While the HMM was being deadheaded, the variant ACTC sample was polymerized to F-actin by incubating the variant in polymerization buffer to a final...

**Figure 5. IVM experimental setup** (A) Diagram of a flow cell. (B) Deadheading gel with very little actin contamination in the supernatant well (#4 S/N). S/N is the abbreviation for sample supernatant and Pel is the abbreviation for the sample pellet

concentration of 50 mM KCl, 25 mM Tris (pH 8.0), 1 mM EGTA, 2 mM MgCl₂, 0.1 mM ATP for 1 hr at room temperature. The variant F-actin was then incubated with rhodamine-phalloidin (Rh-Ph, Fisher) at a 1:2 molar ratio for over 3 hrs at 4 °C. Before the experiment started, serial
dilutions of the Rh-Ph F-actin were made from 10x to 10,000x. These serial dilutions limited the number of actin filaments visualized under the microscope to 30-50 filaments in a field of view. The flow cell was set up first with the addition of 10 μL deadheaded HMM, followed by 10 μL of 1xABSA (1xAB with 0.1 mg/mL BSA) to block any unbound sites on the nitrocellulose coverslip. The 1xABSA wash was followed by 10 μL of 1xAB wash to remove any residual BSA. Ten μL of diluted Rh-Ph F-actin was added to the flow cell, and the number of filaments were inspected under the fluorescence microscope. Once the correct dilution of Rh-Ph F-actin was added, the flow cell was washed with 10 μL of 1x AB, removing any unbound filaments. Finally, 10 μL of 300 nM Tm and Tn mix was added to the flow cell, followed by a final 10 μL 1xAB wash. Before data collection was started, the regulation of the variant filaments was tested using pCa 10. Well-regulated filaments displayed no movement at pCa 10; if movement was observed, another volume of 300 nM Tm and Tn mix was added and washed with 1xAB buffer. This method for producing RTFs within the flow cell was adapted from Viswanathan et al. (2015). Once RTFs were formed within the flow cell, motility buffer (1xAB with the addition of pCa) was flowed through and 45 sec videos were collected in triplicate for each pCa (a total of 33 videos were collect for each IVM experiment).

All IVM videos were collected using a Zeiss Axiovert 200M (Zeiss, Jena, Germany) microscope and the RTFs were visualized at 100x oil-immersed magnification using the Texas Red (510 nm) filter. The RTF movement was captured using a digital CCD ORCA-R2 C10600 camera (Hamamatsu, Middlesex, NJ) controlled by the Volocity 6.3 software (Quorum Technologies, Lewes, UK) at a frame rate of 2.48 frames/sec.
2.5.1 Data collection protocol

IVM is a single-molecule assay, which permits observation of the physical changes that occur with variant RTFs. To accurately measure the changes in single molecule interactions, the total number of filaments was 30 to 50. The optimal dilution was determined visually for each flow cell before a video was collected. Each pCa was collected in triplicate, ensuring that the patch of filaments had an even distribution, and the best representative movement was selected. Observations were made first with the lowest pCa, and then proceeded with progressively higher pCa, making a new slide when the filament contrast and number decreased due to each pCa wash.

2.5.2 Video Analysis

Once the videos were collected for each variant RTF, they were analyzed manually using ImageJ v1.4.3.67 (NIH, Bethesda, MD). The videos were first converted to .tiff files, and their color was changed to gray scale to improve the contrast of each filament. The movement of each RTF was classified in two ways. Method A measured the percentage of filaments moving in a video, while Method B determined the average velocity of the filaments. For Method A, 10 filaments were randomly selected per movie and their movement logged in a spreadsheet as either moving or not moving. Filament movement was logged in triplicate for each pCa, and the data compiled and the mean average movement at each pCa plotted against the corresponding pCa value.

For Method B, the starting location of filament was selected using a line tool. The end of the filament after 10 frames was then selected, drawing a line between the start
and the end of the filament path. The “measure” function within ImageJ was used to measure the distance travelled by each filament. This information was then compiled on an Excel spreadsheet, where the average velocity of each filament was calculated by dividing the length travelled by each filament by the total time over 10 frames. The length was converted from pixel number to nm where the size of each pixel was 89.46 nm. This distance in nm was then divided by the total time over 10 frames (4.032 seconds), providing the velocity of each filament. Averaging the velocities of 10 filaments for each of three videos yields the average velocity of each pCa which was used to plot a pCa curve.

2.6 Data Management

The Dawson lab utilizes a combination of cloud and physical storage for archiving all lab data. All the data and notes pertaining to the RTF ATPase assay have been stored on Confluence (www.atlassian.net/greenpuppy). The .txt files containing the absorbance values, the .xlsx files with the standard curve and data analysis, as well as the. pzf GraphPad Prism 5 files with the plotted pCa curves are also saved on Confluence. Each Confluence page is dated and can be found in the ATPase section of my page under the ATPase log header.

The raw data for the IVM assay was archived on two external hard drives. The .tiff files were compiled in folders according to each experiment date, and each folder was divided between two My Book external hard drives (Western Digital, San Jose) stored in the Dawson lab support room (Room 2223B in the Science Complex). The experimental log, the filament selections (.jpg) for method A, coordinates files (.xls) for method B, and data analysis spreadsheets (.xlsx) are all on my Confluence page in the IVM log.
3. Results

3.1 Purification of ACTC from Harvested Cell Pellets

ACTC proteins were purified from each of the harvested Sf21 baculovirus cell pellets obtained from 500 mL cell culture (Figure 6). The yield for each of these pellets was about 2 mg of ACTC proteins per pellet. This yield was lower than the previously stated amount of 2.20 to 4.25 mg of purified protein in 500 mL cell pellets51.

3.2 Method A Analysis of IVM Videos

I compared the percentage movement of F90Δ or H88Y RTFs against each other and WTrec RTFs. These data were graphed in two ways: the absolute filament movements (AFM) are shown on the left of Figure 7 (A1, B1 and C1), and normalized percentage of filament movement (NFM) are presented in the right panel (A2, B2 and C2).

3.2.1 Comparison Between H88Y and WTrec RTFs

The curves for H88Y RTFs (Figure 7A1 and 7A2) show an increase in the pCa\textsubscript{50} compared to WTrec RTFs. H88Y RTFs have an AFM pCa\textsubscript{50} of 7.05±0.08 and NFM pCa\textsubscript{50} of 7.00±0.06, compared to the WTrec RTFs which have an AFM pCa\textsubscript{50} of 6.61±0.05 and NFM pCa\textsubscript{50} of 6.60±0.03.

3.2.2 Comparison Between F90Δ and WTrec RTFs

Like H88Y, F90Δ RTFs (Figure 7B1 and 7B2) also show an increase in pCa\textsubscript{50} compared to WTrec RTFs. F90Δ RTFs have an AFM pCa\textsubscript{50} of 6.99±0.08 and NFM pCa\textsubscript{50} of 6.92±0.05, (WTrec RTFs have an AFM pCa\textsubscript{50} of 6.61±0.05 and NFM pCa\textsubscript{50} of 6.60±0.03).
Figure 6. Progress of purification gel for ACTC.
Figure 7. Method A data comparing F90Δ and H88Y RTFs to WTrec RTFs. These are the Method A data collected using IVM. Each curve was plotted using GraphPad Prism 5 using the log response-four parameter fit. The curves on A1, B1 and C1, show the total number of filaments moving out of the 10 randomly selected filaments for each video. A2, B2, and C2 show the normalized values for the percentage of moving filaments, for a better visualization of the shift in pCa_{50}. C1 and C2, show the compiled data, and the F90Δ and
H88Y RTFs show a leftward shift in pCa\textsubscript{50} relative to WTrec RTFs (pCa\textsubscript{50} of 6.99, 7.05 and 6.61 respectively). 7C1 and 7C2 are shown to compare the changes in pCa\textsubscript{50} between F90\Delta and H88Y RTFs.

3.2.3 Comparison Between M-Class Variants

Figure 7C1 and 7C2 show both variant curves on the same plot. F90\Delta and H88Y both have similar higher pCa\textsubscript{50} values. This shows that there is a meaningful difference between the pCa\textsubscript{50} of both M-class variant RTFs compared to WTrec (further discussed further in section 4.4.1).

3.3 Method B Analysis of IVM Videos

I compared the absolute and normalized velocities of F90\Delta or H88Y RTFs against each other and WTrec RTFs. The pCa\textsubscript{50} value of each of these variants shows the response of each variant’s velocity to the Ca\textsuperscript{2+} concentration. These data will be used to determine the extent of the changes in regulation for each variant. The absolute velocity (AV) curves are to the left of Figure 8) (A1, B1 and C1) while the percentage of maximal velocity (%MV) are shown on the right (A2, B2 and C2).

3.3.1 Comparison Between the Velocities of H88Y and WTrec RTFs

Figure 8A1 shows the AV curve of H88Y versus WTrec RTFs, and there is a slight leftward shift in the pCa\textsubscript{50} (N=3, pCa\textsubscript{50}=6.91±0.12 versus N=3, pCa\textsubscript{50}=6.88±0.15). These data show that there is an increase in the Ca\textsuperscript{2+} sensitivity for H88Y RTFs. The maximum velocity of H88Y RTFs is slightly higher than that of WTrec RTFs, where the \(v_{\text{max}}\) for H88Y RTFs is 5.14 \(\mu\text{m/s}\) while the WTrec RTFs have a \(v_{\text{max}}\) of 3.44 \(\mu\text{m/s}\). H88Y RTFs have a slight basal velocity of \(v_{\text{min}}\) of 0.7 \(\mu\text{m/s}\), where WTrec had no basal velocity. The % MV curves confirm that H88Y has a higher pCa\textsubscript{50} than WTrec RTFs, showing once more that it has higher Ca\textsuperscript{2+} sensitivity.
Figure 8. Method B data comparing F90Δ and H88Y RTFs to WTrec RTFs.

Method B data collected using IVM. Each curve was plotted like the Method A data. Curves A1, B1 and C1, show the velocities of 10 randomly selected filaments for each of the variants. A2,
B2 and C2 present the % maximal velocity, showing the normalized data for each variant. The AV data shows that there is a very slight rightward shift of the pCa_{50} for H88Y (N=3, pCa_{50}=6.91±0.12), while F90Δ (N=3, pCa_{50}=6.77±0.19) has a leftward shift of pCa_{50} compared to WTrec (N=3, pCa_{50}=6.88±0.15) RTFs. 8C1 and 8C2 are shown to compare the changes in pCa_{50} between F90Δ and H88Y RTFs.

3.3.2 Comparison Between the Velocities of F90Δ and WTrec RTFs

The pCa_{50} determined from the AV curves of F90Δ RTFs and WTrec RTFs were N=3, 6.77±0.19 and N=3, 6.88±0.15 respectively. The maximum velocity for F90Δ was also higher at 4.00 μm/s, compared to WTrec, which has a maximum velocity of 3.44 μm/s. The % MV shows that F90Δ RTFs had a significantly higher velocity (p=0.05, using Kruskal-Wallis test) compared to WTrec RTFs even though they had similar pCa_{50} values (N=3, 6.91 and N=3, 6.88 respectively).

3.3.3 Comparing the Velocities of the M-Class Variants

Figure 8C1 and 8C2 show a comparison of both M-class variant pCa curves. It is evident that there is a more significant pCa_{50} shift leftward for the H88Y variant compared to F90Δ RTFs. This change in velocity of H88Y showed that the actomyosin interactions are directly affected by this variant since the regulatory proteins should not have a direct impact on the in vitro RTF velocity. This observation will be further examined in the discussion.

3.3.4 Variant RTF Filament Velocity Distribution

Figure 9 shows the distribution of the individual filament velocities for each of the variant RTFs. This figure was used to show the variation of velocities of each set of RTFs, which helped explain why the Method B data for each variant had a large SEM. A total of 1,012 filaments were measured for WTrec, 1,001 for F90Δ and 1,012 for H88Y. These represent three
biological replicates of each variant, with a total of 3,025 filaments analyzed. The resulting scatter-plots show that there is a large amount of variation in the filament movement at lower pCa, compared to the higher pCa.

A) 

B) 

C)
Figure 9. Distribution of RTF velocities at each pCa. These scatter plots show the individual velocities for each set of variant RTFs. Each point represents the velocity of a single filament, and the scatter plots were made using the vertical column-scatter graph function in GraphPad Prism 5 (San Diego, California). The total number of filaments for each variant are as follows; WTrec RTF (1,012, N=3), F90Δ RTFs (1,001, N=3) and H88Y RTFs (1,012, N=3)
3.4 Preliminary RTF ATPase Data for F90Δ and H88Y

Only a single biological replicate was successfully tested for each of the M-class variants along with WTrec RTFs (Figure 10). These data have been presented to show the general trend observed with this enzymatic assay. The figures on the right (Figure 10A1, B1 and C1), show the absolute myosin rate (μM/min) against the pCa, and the figures on the left (Figure 10A2, B2 and C2), show the normalized ATPase activity. The absolute myosin rate curves show differences in the maximal and minimal activities for each of the variants, while the normalized ATPase activity emphasizes the pCa50 values for each of the variant RTFs.

The F90Δ RTFs have a pCa50 of 7.25±0.02 which is higher than that of WTrec RTFs, which have a pCa50 of 7.04±0.06 (Figure 10B1). This difference is further highlighted in the normalized ATPase activity curves, where F90Δ RTFs have pCa50=7.27±0.01756 while WTrec RTFs have a pCa50 of 7.00±0.05 (Figure 10B2). This relative increase in pCa50 for F90Δ RTFs shows that the F90Δ variant could contribute to an increase in calcium sensitivity. H88Y RTFs do not show any relative changes in calcium sensitivity compared to WTrec RTFs. H88Y has a pCa50 of 6.94±0.08, which is similar to WTrec RTFs with a pCa50 of 7.04±0.06 (Figure 10B1). A similar trend is also observed in the absolute pCa50 of H88Y when compared to WTrec, with each of them having pCa50 of 6.93±0.06 and 7.00±0.05 respectively (Figure 10A2). Each of the points within the curves were determined in triplicate, but only a single biological replicate was obtained for each of the variant RTFs. It is therefore not possible to comment on the significance of these data. However, these preliminary data show a trend where the F90Δ RTFs have a higher pCa50 than WTrec, which
has been observed in the IVM Method A data as well. Future experiments on these mutants will reinforce this trend and improve the significance to these data.
Figure 10. Preliminary pCa curves of RTF ATPase assay. These pCa curves show the enzymatic response of each of the variant RTFs, at different pCa. The shift of each of the variant RTFs as well as the overall myosin rate can be observed from each of these curves. Each curve was plotted using GraphPad Prism 5 (San Diego, California) using the log response-four parameter fit. In brief the F90Δ RTFs show a right-ward shift, with a pCa of 7.27±0.02 (N=1), while H88Y RTFs had a similar pCa to WTrec RTFs (pCa of 6.93±0.06, N=1 and 7.00±0.05, N=1 respectively). Note that each of the pCa points within these curves were done in triplicate,
and the error bars represent this differences in these values. 10C1 and 10C2 are shown to compare the changes in pCa$_{50}$ between F90Δ and H88Y RTFs.
Table 1. pCa\textsubscript{50} values for RTF variants. Each of these values was found using Graph Pad Prism 5, where these data were fitted to a log versus response-four parameter curve. The pCa\textsubscript{50} values for the absolute as well as normalized data have both been added for each variant. The data was entered into SPSS (Richmond, CA) to obtain the average pCa\textsubscript{50} of each variant, and a Kruskal-Wallis test with a Bonferroni correction was made to each data set to determine the significance of the data. These data were not significant but the Method A data (*) show a trend towards significance, since the p value is 0.066, and the effect size of the data is high (0.57).

<table>
<thead>
<tr>
<th>ACTC Variant</th>
<th>IVM Assay- Method A</th>
<th>IVM Assay Method B</th>
<th>RTF ATPase Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absolute</td>
<td>Normalized</td>
<td>Absolute</td>
</tr>
<tr>
<td>WTrec</td>
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<td>6.60±0.03</td>
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<tr>
<td>F90del</td>
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</tr>
<tr>
<td>H88Y</td>
<td>*7.05±0.08</td>
<td>7.00±0.06</td>
<td>6.74±0.14</td>
</tr>
</tbody>
</table>
4. Discussion

4.1 Purification of Recombinant F90Δ and H88Y

The ACTC variant proteins expressed using the Sf21/baculovirus system were purified successfully using the affinity and anion exchange protocol outlined in section 2.3.4. The purified ACTC variants were tested by Liu (2016) using a Western blot with an α-actin (5C5) mouse monoclonal primary antibody. SDS-PAGE analyses were performed for every purification to ensure the purity of the final sample. The progress of the resulting acrylamide gels has been scanned and uploaded onto my Confluence page under the Large-Scale purification section.

4.2 Variations in Producing RTFs

Two methods for RTF production have been used and each of these has some advantages and disadvantages associated with them. The first method for producing RTFs was outlined and tested by Liu (2016). This method is detailed in section 2.4.1 and, in brief, involves mixing 22.2 μM ACTC, 9.43 μM Tm and 9.43 μM Tn, followed by a 2-day dialysis in 1xABB (formula in section 2.4.1). This method was utilized by Liu (2016) in both the RTF ATPase and IVM assays, which were used to test E99K and R95C RTFs. In contrast, I have only used this method for the RTF ATPase since this assay requires the use of a consistent concentration of each protein. This method is followed to ensure that the results are accurate and reproducible, since differences in protein concentrations could lead to changes in pCa50 values for the variant RTFs. This method of producing RTFs has a significant drawback: it requires the addition of all the proteins in a single mixture. The presence of all the proteins within a single mixture could cause issues with the experiments, as all proteins regardless of their functionality are present within a
single mixture. The presence of non-functional protein with the RTF mix could result in a significant proportion of the variant ACTC remaining unregulated, which has been shown to negatively affect the pCa50 values of the variants. The unregulated F-actin can activate myosin ATPase activity without the need for any Ca2+, which can create a false positive since RTFs at low Ca2+ concentrations would remain blocked by Tm and Tn. High levels of Pi production has been a key issue affecting the RTF ATPase assay results, since most of these assays have failed due to there being no change in the RTF activity levels at different pCa. This RTF mix could also have implications on IVM assays where a significant amount of unregulated F-actin could create false positives that could be confused for increased calcium sensitivity for the variant RTFs. However, the protocol for the IVM assay involves the washing of unbound protein out of the flow cell between each protein addition step (outlined in section 2.4.3). These washes ensure that any non-functional proteins are washed out of the flow cell, reducing the possibility of false positives. This washing step explains why Liu (2016) and I obtained the same WTrec pCa50 for IVM method A (6.64±0.11 and 6.61±0.05 respectively).

The issues experienced due to the 2-day dialysis method of RTFs led to the development of a second method for IVM, which is outlined in section 2.4.3. The RTFs in this method were produced by flowing 300 nM of Tn and Tm over variant F-actin. Since the F-actin is diluted 10,000-fold, the addition of 300 nM Tn and Tm saturates the flow cell with these proteins. This step is followed by a wash with 1xAB (section 2.4.3), which washes out any unbound, non-functional protein. This method was adapted from a protocol developed by Viswanathan et al. (2015), with the goal of reducing the total
amount of ACTC prepared for IVM assays, from micrograms to nanogram. The initial method for RTF production used approximately 1 mg of ACTC, with the flow cell method for RTF production using 2 ng. This is an improvement over the 2-day dialysis method for producing RTFs, since only functional RTFs are present in the flow cell. This method allowed me to successfully gather more replicates for the RTF IVM assays but this method of RTF production cannot be used for the RTF ATPase assay. Due to the RTF ATPase assay’s requirement for specific protein concentrations, it was not possible to adapt the quick RTF forming method for it. The best way to improve the RTF production procedure may be to eliminate any excess unbound proteins before running this assay. This can be done by eliminating any non-functional unbound protein by centrifuging the samples, and ensure the pellet containing RTFs is used for the assay only. One major complication that may arise from this centrifugation step is that the final ACTC concentration has to be determined accurately before the experiment is performed.

4.3 Controls Used for IVM and RTF ATPase Assays

The F90Δ and H88Y RTF’s pCa50 values from ATPase assays were compared to WTrec RTFs. Previous researchers in the Dawson lab used bovine ACTC as a control since it has a 100% sequence identity to human ACTC and it is available at high concentration after tissue purification. Further testing of bovine RTF ATPase activity against that of WTrec RTFs has shown a clear difference in the pCa50 values of these two proteins51. These differences prompted the use of WTrec ACTC rather than bovine ACTC as the comparator for recombinant ACTC variants, yielding consistent results for each assay.

As stated earlier, the pCa50 values for WTrec in IVM method A are similar to Liu (2016) with my pCa50 value being 6.61±0.05 while his WTrec pCa50 value is 6.64±0.11. The method B
data analysis is quite different, however with my WTrec pCa$_{50}$ being 6.88±0.15, while Liu (2016) WTrec pCa$_{50}$ is 6.51±0.02. The RTF ATPase data cannot be reliably compared between my work and Liu (2016) work since I only have one biological replicate. These differences values obtained by method B could be attributed to the different methods of RTF production for the IVM assays, since Liu (2016) used leftover RTFs from the RTF ATPase assay (2-day dialysis method of producing RTFs), while I used the newer method of binding Tn and Tm to F-actin directly in the flow cell. This may have led to Liu (2016) lower WTrec Ca$^{2+}$ sensitivity, since there is a possibility that there is a significantly higher proportion of non-functional Tn complex present. This higher proportion of non-functional Tn could lead to a higher concentration of Ca$^{2+}$ being required to shift tropomyosin and activate the myosin cross-bridge activity. The use of WTrec ACTC rather than bovine ACTC was also implemented to ensure that all variant comparisons are more meaningful, since bovine ACTC involves the use of tissue purified protein rather than recombinantly produced proteins, which introduce differences in post-translational modification.

4.4 H88Y and F90Δ IVM Results

The shift in pCa$_{50}$ values for the each of these variants was different for each method of analysis. Method A results for both variants showed an increase in Ca$^{2+}$ sensitivity relative to WTrec RTFs. The method B data, on the other hand, did not show a significant trend in the pCa$_{50}$ of either one of these variants when compared to WTrec RTFs. I will go into further detail about the pCa$_{50}$ values for each method separately in the next two subsections.
4.4.1 Increased pCa$_{50}$ Observed in Method A

The percentage of motility relates the proportion of RTFs moving in the presence of Ca$^{2+}$ a particular concentration. The pCa curves generated with this method of data analysis allow us to visualize the level of RTF activation in response to the concentration of free calcium within the buffer. The higher the Ca$^{2+}$ concentration, the greater the level of expected RTF movement. I observed an increase in the percentage of moving filaments for both H88Y and F90Δ RTFs at lower pCa values, relative to WTrec RTFs. This increase was evident in the pCa$_{50}$ of each of these variant curves as summarized in Table 1. These were higher than the WTrec RTFs, which had an AFM pCa$_{50}$ of 6.61±0.05 and an NFM pCa$_{50}$ of 6.60±0.03. A Kruskal-Wallis non-parametric test was performed on the pCa$_{50}$ values using IBM SPSS 25, and it had a p value of 0.066, which is just shy of 0.05 required for the data to show a significant difference. However, there were only three biological replicates for each variant pCa$_{50}$, which may have resulted in this p value being slightly higher. To test whether the increase in pCa$_{50}$ was meaningful, I tested the effect size of the variant RTF’s pCa$_{50}$ values. The effect size was using the effect size formula$^{58}$ and I found that the $\eta^2$ value for these data was quite high (0.57). This showed that while the increase in the pCa$_{50}$ values of F90Δ and H88Y did not have any significant difference, the high effect size meant that the magnitude of change of the pCa$_{50}$ was meaningful.

The higher Ca$^{2+}$ sensitivity for H88Y and F90Δ variant RTFs shown in the data presented here translated into more cross-bridge interactions forming at lower Ca$^{2+}$ concentrations. The higher pCa$_{50}$ values for both H88Y and F90Δ RTFs show that these M-class variants alter thin filament regulation, which may be linked to the occurrence of hypertrophy in early-onset HCM. I
expected to see this increase in Ca\(^{2+}\) sensitivity, since other variants involved in the development of HCM cause a similar increase in pCa\(_{50}\). It is also clear that these variants’ involvement in early-onset HCM might be attributed to an increase in the frequency of cross-bridge interactions within the cardiac thin filament.

4.4.2 Increased Maximal Velocity of H88Y and F90Δ

The method B data did not show any significant changes in pCa\(_{50}\) for the H88Y and F90Δ variants when compared to WTrec (p=0.83). Table 1 shows that both the absolute pCa\(_{50}\) for H88Y and F90Δ variant RTFs do not show any significant difference. The \(v_{\text{max}}\) of both variants, however, was higher for H88Y and F90Δ RTFs compared to WTrec RTFs. While the \(v_{\text{max}}\) did not show any significant difference (p=0.051), which occurred due to the lower N value, the effect size of the change was high (0.659). The higher \(v_{\text{max}}\) values show a trend towards significance in this case.

An increase in the maximal velocity for the RTFs could represent a faster rate of cross-bridge formation caused by these variants. This increase could have implications on the force production and transmission within the cardiac muscle. These changes in velocity are independent of actomyosin regulation. While the lack of changes in the pCa\(_{50}\) of the Method B of H88Y and F90Δ goes against my hypothesis, the changes in \(v_{\text{max}}\) could be indicative of an alternate pathway that may lead to the development of HCM.

4.5 Preliminary Results of the RTF ATPase Assay

As stated in the previous section, only one biological replicate was obtained for each variant (WTrec, H88Y and F90Δ) due to potential issues with the RTF formation for
this assay. These preliminary results can be used to predict a general trend that may be observed once additional RTF ATPase assays are completed for these variants. F90Δ has a pCa$_{50}$ value of 7.25±0.02, while WTrec had a pCa$_{50}$ of 7.04±0.06. These higher pCa$_{50}$ values could show us that F90Δ ACTC could lead to an increase in Ca$^{2+}$ sensitivity by altering the cross-bridge regulation by Tn and Tm. This possibility aligns well with my hypothesis, since F90Δ is involved in cardiac hypertrophy, which may be explained by an increase in Ca$^{2+}$ sensitivity due to altered cross-bridge regulation. H88Y, on the other hand, does not show any significant trend towards increased Ca$^{2+}$ sensitivity since its pCa$_{50}$ value is 6.94±0.08, similar to WTrec RTFs pCa$_{50}$ of 7.04±0.06. While these trends are not conclusive, it is interesting to note that these variants found in similar forms of early-onset HCM have different RTF ATPase activity.

However, it is important to note that while both variants are found in early-onset forms of HCM, the H88Y mutation results in a more benign amino acid substitution, rather than the deletion of F90Δ. This difference may result in a reduced effect on the enzyme kinetics due to H88Y variants, which has a similar pCa$_{50}$ value in this assay when compared to WTrec RTFs.

4.6 Implications of IVM Results

The IVM and preliminary RTF ATPase results examine the effects of ACTC variants within RTFs as they interact with myosin. The goal of my study was to understand the effects that these variants have on regulation, by measuring the changes of RTF calcium sensitivity using multiple assays. It is important to note, however, that the Ca$^{2+}$ sensitivity changes in my experiments do not necessarily provide the same information as do other model systems. We use a highly simplified structure in the form of RTFs, and the changes in Ca$^{2+}$ sensitivity are used to determine how the regulatory complex reacts. We are therefore unable to compare these changes
in Ca\(^{2+}\) sensitivity directly to other models such as reconstituted myofibres\(^{59}\). The key advantage to using RTFs, however, is that they allow us to focus on changes in specific sarcomeric proteins without complicating factors of whole-cell systems\(^{60}\).

Performing experiments on RTFs may seem novel and unreliable, but several other researchers in this field use a similar approach. The reason for this is that studying the functional changes of each sarcomeric protein within functioning heart tissue is still not possible. It is also not possible to study individually the structural and functional changes of the thin filament protein variants. A majority of these assays rely on the actin-activated myosin ATPase activity to quantify changes that occur due to thin filament variant proteins involved in the development of HCM\(^{61}\). Apart from measuring actomyosin interactions using the actin-activated myosin ATPase, the regulatory changes are studied using changes in Ca\(^{2+}\) sensitivity which allow researchers to determine whether there is a shift from normal sarcomere function. However, measuring the changes in Ca\(^{2+}\) does not provide any information which can be linked to molecular pathways that may be directly involved in the progression of HCM.

My observations of Ca\(^{2+}\) sensitivity changes in H88Y and F90Δ RTFs show us that there is a shift in sarcomere regulation due to these variants. It is safe to assume that the results reported in the previous sections influence the force production and transmission within the sarcomere. To understand how the force production may be affected within the cardiac sarcomere, I will briefly describe the force development model\(^{62}\). This model assigns parameters for the development of force by cross-bridge interactions, and changes to these physical parameters have predicted effects on the
overall force production by the sarcomere. The total force by an individual sarcomere is the **force of the ensemble** ($F_{EN}$), described by equation 1:

$$F_{EN} = f \cdot r \cdot N_T$$  \hspace{1cm} (Eq. 1)

where $f$ is the force generated per single actomyosin molecular interaction, $r$ is the duty ratio (proportion of the myosin ATPase cycle spent bound to actin), and $N_T$ is the total number of myosin heads interacting with the actin filament in the sarcomere. $N_T$ is kept constant when the molecular interactions of actin and myosin are being studied, while the changes in duty ratio and force per actomyosin interaction represent a proportional change in the ensemble force. It is important to keep in mind that the fundamental unit of contraction is a single sarcomere. These changes in ensemble force are based on the assumption that these variants affect the force production within a single sarcomere, and the combined changes in individual sarcomere force production may lead to this dysfunction. These variations in the ensemble force lead to dysfunction in cardiomyocyte contractility, which may cause the development of HCM.

Based on the results from IVM method A analysis, there is an increase in the percentage of RTF movement at lower Ca$^{2+}$ concentrations. With regards to the $F_{EN}$ equation, the increased pCa$_{50}$ could result in a higher $N_T$ at lower Ca$^{2+}$ that could result in increased force production by the cardiac sarcomere. The increased $v_{max}$ observed in the IVM method B analysis could indicate that there is rapid hydrolysis of ATP. This rapid hydrolysis of ATP, could represent an increase in the $k_{cat}$ of myosin ATPase, which may affect the $r$. The $r$ is calculated using equation 2:

$$r = \delta \cdot k_{cat} / v_0$$  \hspace{1cm} (Eq. 2)
where \( \delta \) is the myosin step size, \( k_{\text{cat}} \) is the turnover rate of the myosin ATPase activity and \( v_0 \) is the velocity of myosin motor. The myosin step size \( \delta \) is generally agreed upon as 5 nm, using laser trapping studies. \( k_{\text{cat}} \) can be directly measured by using a myosin ATPase assay quantifying the rate of ATP hydrolysis due to actin-myosin cross-bridge formation.

In this case, the increase in \( k_{\text{cat}} \) is observed in the Liu (2016) ATPase data could be cancelled out by the higher \( v_0 \) that is observed in IVM method B analysis, where the \( v_{\text{max}} \) of H88Y and F90Δ RTFs are higher than WTrec RTFs. This change would not cause any change in the \( r \) value, meaning that the altered \( N_T \) is the only parameter that could increase the \( F_{\text{EN}} \), altering the normal force production within the heart, increasing the cardiac stress on the heart that could be responsible for ventricular hypertrophy.

The RTF ATPase data of H88Y and F90Δ RTFs shows a different effect for each variant, however, making it important to understand the reason for these differences. F90Δ RTFs show a trend of increased \( \text{Ca}^{2+} \) sensitivity, as they have a higher pCa50 in the method A analysis of the IVM assay as well as the preliminary results of the RTF ATPase assay (Table 1), relative to the WTrec RTFs. They did not, however, show any difference in pCa50 of the method B analysis of IVM, but did show a significant difference in their \( v_{\text{max}} \) compared to WTrec RTFs. Deletion of F90 altogether can alter the register of a key helix involved in interactions between myosin and actin. Arg95 and Glu99 are most likely affected, altering actomyosin interactions directly. Apart from the known changes in actomyosin interactions, my study of F90Δ RTFs takes the knowledge of these changes further, as the increased \( \text{Ca}^{2+} \) sensitivity, relating to combined filament movement and myosin ATPase activity, show that thin filament regulation has also been
affected. While the deletion of F90 does not affect any sites known to play a role in Tn and Tm interactions, these data show that direct Tm and Tn binding need to be tested further in the context of F90Δ RTFs.

While H88Y does not show any significant trends for the RTF ATPase assays and IVM method B analysis, it does show an increase in Ca^{2+} for the IVM method A analysis. This change in regulation reinforces the fact that the replacement of His88 with tyrosine results in a variant capable of affecting cross-bridge regulation. The histidine would have a partially protonated imidazole side chain, which would be capable of stabilizing folded structures through pi-stacking interactions. The tyrosine substitution provides an aromatic side chain, which could participating in similar pi-stacking interactions. The histidine side chain on the other hand is polar and positively charged, while the substituted tyrosine side chain is neutral charge. This shift could have an impact on regulatory interactions near the myosin binding site, which could explain the shift in pCa_{50} for H88Y RTFs as well as the increased v_{max} compared to WTrec RTFs.

4.7 Comparing Variants to Other HCM Mutations

It is important to compare the results from this study on F90Δ and H88Y ACTC variants to other M-class variants. The Liu (2016) thesis presents the regulatory changes that occur due to E99K and R95C RTFs, with E99K ACTC being one of the best-established. He showed that both E99K and R95C RTFs show significant decreases in Ca^{2+} sensitivity using the IVM assay. These data do not align well with the prevailing hypothesis, since most of the variants involved in the development of HCM lead to an increase or no change in the Ca^{2+} sensitivity of the variant. When compared to E99K and R95C variant data, there is a trend towards increased Ca^{2+} sensitivity for F90Δ and H88Y IVM method A analysis. Compared to the E99K and R95C
RTF data, this study shows a similar trend to other research on M-class variants\textsuperscript{64}. However, one should not fully disregard the E99K and R95C data since they were able to show significant differences. These differences in results could be attributed to two important factors within each experiment. One of the key differences between the M-class data collected for E99K and R95C was the method of RTF production. As stated in section 4.2, Liu (2016) used RTFs from the RTF ATPase method, rather than the newer flow cell method that I used. This may have led to the decreased pCa\textsubscript{50} for these variant RTFs. The other reason for this lower pCa\textsubscript{50} could be linked to the actual site of each mutation that leads to a reduction in the Ca\textsuperscript{2+} sensitivity of these variant RTFs. While the increase in pCa\textsubscript{50} has been observed in all HCM variants, the models used to study these changes were different. It is therefore highly possible that in RTF systems, E99K and R95C are able to disrupt Tm and Tn in such a way that they subsequently reduce the Ca\textsuperscript{2+} sensitivity, while the site of mutation leading to the F90\Delta variant may cause an increase in Ca\textsuperscript{2+} sensitivity, making it a gain-of-function mutation.

4.8 Summary of Discussion

There was a clear trend of increased Ca\textsuperscript{2+} sensitivity for F90\Delta RTFs in both assays. However, the lack of change in the Ca\textsuperscript{2+} sensitivity of H88Y shows that the prevailing hypothesis, as well as the working hypothesis presented herein, needs to be refined. Apart from H88Y, there are other ACTC mutations that do not cause an increase in calcium sensitivity, such as A331P. These variants are often omitted from reviews, which try to draw a clear connection between the increase in Ca\textsuperscript{2+} sensitivity with the subsequent development of hypertrophy present in HCM.
While these results do not allow us to draw a clear link between all forms of HCM variants, it is quite clear that the presence of these variants contribute to the HCM phenotype. It is highly probable that these mutations are able to act together with other thick and thin filament proteins to lead to early-onset variants. Apart from F90Δ and H88Y ACTC variants, several other variant sarcomeric proteins may be present within a patient. The next step would be to determine a clear pathway between the development of this disease by looking at the interactions of other variant proteins with ACTC variants. The IVM assay allows us to complex several different proteins to F-actin on a flow cell. By altering the protocol, one could add several other sarcomeric protein variants to the flow cell, observing changes in the RTFs. However, it is not feasible for a single lab to carry out all of this research, since the recombinant production and purification of these variant proteins would likely take several years to perfect.

It is also important to focus on the development of assays that can report on the binding and conformational changes of the Tn and Tm complex with variant thin filament proteins. A key hurdle within this research is that there is no direct measure for studying the function of thin filaments and their regulatory proteins. The only reliable method is to study the Ca²⁺ sensitivity of the variants, which is analyzed by understanding how the myosin motor protein reacts. While the Ca²⁺ sensitivity is frequently used within the field, it has several major issues. One of the key issues with this assay is that the Ca²⁺ sensitivity changes can only show relative changes away from equilibrium. It is, however, unable to report back on the specific protein components which may be involved in the progression of disease.

5. Conclusion
This study shows that there are unique changes in regulation of F90Δ and H88Y RTFs. These differences in Ca\(^{2+}\) sensitivity across different assays further emphasize that mutations found in the same sub-domain can have entirely different effects on actomyosin interactions. Each of these point mutations demonstrate different functional changes, and as a result, indicate a different pathway in the development of HCM. It is interesting to note that while the severity of changes related to F90Δ and H88Y are quite different, their resulting phenotypes are similar. This fact demonstrates that not a single point mutation, but several sarcomeric dysfunctions are involved in the progression of hypertrophy observed in HCM.

Several researchers have stated that energy depletion, force transmission, abnormal calcium homeostasis and dysfunctional vascular endothelium are potentially involved in the development of HCM. I believe that multiple issues with force transmission and energy depletion are involved in the progression of HCM due to ACTC variants. As observed with the F90Δ variant, an increased Ca\(^{2+}\) sensitivity and \(v_{\text{max}}\) have a direct effect in the regulation and overall force production of the sarcomere. The prolonged effect of these hypercontractile conditions could lead to the development of hypertrophy. Hypertrophy can be exacerbated by the depletion of cardiomyocyte energy production, due to this gain of function mutation. The H88Y RTFs, on the other hand, only show an increased Ca\(^{2+}\) sensitivity in IVM method A, showing no change in Ca\(^{2+}\) in the RTF ATPase and IVM method B data, while showing a significant increase in \(v_{\text{max}}\) that indicates that the force transduction is directly affected by the variant. This change in force transduction may lead to the development of hypertrophy in a similar way to F90Δ.
While this study does not provide any specifics regarding the effects of these variants, it does provide us with a better understanding of the M-class ACTC variants as a whole. This study shows us that even variants with similar phenotypes, and present within the same ACTC subdomain, have different effects on regulation, highlighting the need for an assay that can determine the function of Tn and Tm without the presence of myosin. Understanding the structural and functional changes caused by ACTC variants would shed light on the mechanisms that lead to the changes in Ca^{2+} sensitivity. A deeper understanding of the molecular changes within the sarcomere would allow us to develop personalized therapeutics that are better able to halt the development of early-onset HCM.
References

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